ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF MERCURY RESISTANT BACTERIA (MRB) FROM SOIL SAMPLES OF INDUSTRIALLY CONTAMINATED AREA OF ROURKELA, ORISSA

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN LIFE SCIENCE

BY:

SINDHUSHREE GIRI
ROLL. NO. 409LS2040

Under the guidance of

Dr. Surajit Das

Assistant Professor

Department of Life Science

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769 008, ORISSA

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Rourkela SINDHUSHREE GIRI

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LIST OF SYMBOLS AND ABREVIATIONS USED

Gm	Gram
hr	Hour
l	Litre
µl	Microlitre
°	Degree
C	Centigrade
ml	mili litre
min	minute
M	Molar
NA	Nutrient Agar
LB	Luria Bertani
MHB	Muller Hinton Broth
MIC	Minimal Inhibitory Concentration
ppm	Parts per million
%	Percentage
THB	Total Heterotrophic Bacteria
MRB	Mercury Resistant Bacteria
+	Positive
-	Negative
No.	Number

1. INTRODUCTION:

SCIENCE IS BUT AN IMAGE OF THE TRUTH.

------ Francis Bacon

Mercury pollution of the environment by mining activities and industrial has resulted in worldwide contamination of large areas of soils and sediments and let to elevated atmospheric mercury levels (Baldwin and Marshall, 1999). Because of lack of suitable cleanup technologies, efforts to deal with polluted sites are directed toward the mechanical removal of contaminated material and its deposition elsewhere. Such processes are costly and often result in remobilization of toxic mercury compounds during the dredging process (Bogdanova et al., 1992). Mercury is one of the most toxic elements as it binds to the sulfhydryl groups of enzymes and proteins, thereby inactivating vital cell functions (Sheffy, 1978). After discharge of mercury into the environment, mercury enters the sediments where it persists for many decades. It is taken up by aquatic organisms in the form of highly toxic methyl mercury and is subsequently biomagnified through the food chain and thus the health of top predators, e.g., birds, fish, seals, and man, is thereby threatened. At high concentrations, mercury vapor inhalation produces acute necrotizing bronchitis and pneumonitis, which is lead to death from respiratory failure. Long term exposure to mercury vapor primarily affects the central nervous system and it also accumulates in kidney tissues, directly causing renal toxicity, including proteinuria or nephritic syndrome. High concentration of Hg^{2+} causes impairment of pulmonary function and kidney, chest pain and dyspnousea (Belton and Gorby, 1995). Therefore, the discharge of mercury into the environment needs to be prevented by efficient and cost-effective end-of-pipe treatment technologies for mercury emitting industries (Kleinert and Degurse, 1972). Purification of areas polluted by heavy metals such as mercury is difficult, because the metals cannot be transformed into harmless elements. Over a few decades, community is devoting concentrated efforts for the treatment and removal of heavy metals in order to face this problem. Various types of technology is available for removing of mercury in water and wastewater including

chemical precipitation, conventional coagulation, reverse osmosis, ultrafiltration, magnetic filtration, ion exchange and activated carbon adsorption and chemical reduction (Wood, 1972). Biological systems have been thought to be adapted for removal of toxic heavy metals. Bioremoval is biological systems for removal of metals ion from polluted water has the potential to achieve greater performance at lower cost than nonbiological wastewater treatment. Developments in the field of environment biotechnology indicate the bacteria, fungi, yeasts and algae can remove heavy metals from aqueous solution by adsorption.

In bacteria resistance to mercury is related to enzymatic reduction of Hg^{2+} to volatile Hg^{0} (Bridges and Zalups, 2004; Rudolfs et al., 2004). Mercury detoxification process originated from mer operon located on either plasmids or transposable elements in the mercury resistant microorganisms (Moreno et al., 2008; kannan and krishnamorthy, 2006). Specific transport of bulk mercury across the cell membrane is achieved by two *mer* operon genes merP and merT, which express cystein-rich protein to deliver ambient mercuric toward intracellular mercuric reductase for subsequent reduction of mercuric ions to volatile Hg^{0} (Hamlett et al., 1992).

Studies (Jensen and Jernelov, 1969; Kudo and Hart, 1974) indicate that many common inorganic and organic mercury compounds which are discharged by industry into public waters settle in bottom muds and are converted into alkyl mercury compounds, i.e., mono methylmercury and dimethylmercury. Even though both inorganic and organic mercury compounds enter natural waters, monoalkyl and dialkyl forms of mercury present the greatest threat to all food chains due to their mobility in water and their solubility in membrane lipids. Mercury present in fish as well as other aquatic organisms is almost entirely in the methyl mercury form.

Up to 97% of the mercury formed in aquatic systems is associated with bed sediments (Jernelov and Martin, 1980), and it can remain biologically available for a long period of time (McDuffie et al., 1976). Methyl mercury is not readily retained by sediments, but it is commonly released into overlying waters where it can be taken up by aquatic

organisms. It accumulates in body fat tissues and biomagnifications occurs as larger animals consume mercury laden prey.

Sediments are largely anaerobic, except for a narrow, oxidized microzone at the sediment water interface (Revesbech et al., 1980). Because most of the mercury in an aquatic system is present in the sediments, the highest concentrations of mercury occur in anaerobic environments.

Some microorganisms in the soils have developed resistance to the different forms of mercury, resistance that is based on the functions encoded by the mercury resistance (*mer*) operon (Barkay et al., 2000). In the *mer*-based system *merP* transfers Hg^{2+} from the periplasm of the cell to *merT*, an inner membrane spanning proton that transports Hg^{2+} into the cell. In the cytoplasm the product of *merA*, the enzyme mercuric reductase, takes Hg^{2+} and reduces it to gaseous Hg^{0}, which readily diffuses across cell membranes and in this way resistance is mediated by the elimination of Hg from the cell's local environment. The *mer* operon is specific for Hg^{2+} although the system can handle methyl mercury with the help of the gene, *merB*, which encodes for the organomercurial lyase (Holtze et al., 2006; Takeuchi et al., 2003). Soil and aquatic bacteria have a well-defined system of coordinated processes that target mercury to escape its toxicity as shown in Fig 1.

The *mer* operon (mercury resistance operon) is widely distributed amongst natural microbial community possessing resistance to mercury. Bacterial adaptation to high concentration of mercury includes the induction of mer operon through the action of the *mer R* (regulator of mer operon). Induction of mer operon by *mer R* results in production of non constitutive mercuric reductase (an oxidoreductase) and organomercurial lyase. Clustered of genes in an operon (i.e. *mer*) allows bacteria to detoxify Hg^{2+} to volatile mercury by enzymatic reduction. After this finding there were several reports of environmental bacteria compounds (Walker and Colwell, 1974; Olson et al., 1979). Previous investigations showed that mercury resistance bacteria have some potential for the treatment of individual effluents containing Hg (II). Moreover, researchers showed

that, this process is also applicable to industrial wastewater of the chloroalkali industry (Caustein et al., 1999).

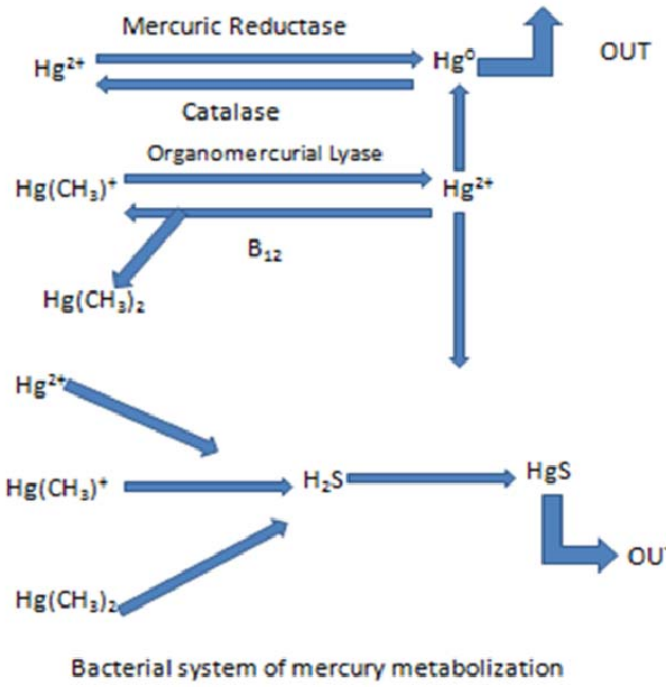


Figure 1. Bacterial system of mercury metabolism(Begley *et al*., 1986)

2. OBJECTIVES AND PLAN OF WORK:

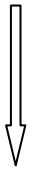
2.1. Objectives

Keeping in mind the above views, present research is based on the following objectives:

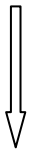
- To isolate and enumerate bacterial populations capable of tolerating mercury from the sediment
- To study the effect of mercuric ion on bacterial growth and determination of minimum inhibitory concentration (MIC)
- To study the growth kinetics of mercury resistant bacteria (MRB)

2.2. Plan of work

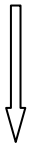
COLLECTION OF SEDIMENT SAMPLE FROM FOUR DIFFERENT SITES (LAGOON INLET) OF ROURKELA STEEL PLANT, ROURKELA



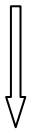
ENUMERATION OF TOTAL HETEROTROPHIC BACTERIA AND MERCURY RESISTANT BACTERIA (MRB) POPULATION (IN 5ppm AND 10ppm $HgCl_2$)



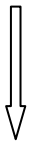
MINIMAL INHIBITORY CONCENTRATION (MIC) TEST



SELECTION OF MRB SHOWING HIGHEST MIC RESULT



BIOCHEMICAL TESTS



GROWTH KINETIC TESTS

3. AREA OF STUDY:

3.1. Rourkela Steel Plant

Rourkela Steel Plant installed dedicated Waste Water Treatment Plant (WWTP) to treat the process waste water generated from most of the units. This waste water along with the storm water collected through the drains(total 10 nos. of drains called outfalls 1 to 10) is discharged to a common nala called Guradih nala which in turn take the total quantity of waste water to a final polishing unit called 'lagoon' which is a shallow oxidation pond. Over the years, the lagoon has got silted thereby shrinking the water spread area of the lagoon. Accordingly, in order to revamp the entire system, RSP entrusted WAPCOS Ltd. to carry out consultancy services for development of stilling basin and desilting of lagoon. Rourkela and its periphery are selected as the potential for the study of environmental pollution due to various industrial activities. The steel industry and other large number of medium industries like cement, rfractories, mining, heavy machinery, fertilizers, explosives, chemicals, distillation, sponge iron mills etc generate various pollutants.

3.2. General Information on Site

The lagoon area is spread in a region of 52 Hectares near Rourkela Steel Plant at Rourkela. For the past 40 years, the lagoon area has been continuously filled up with the discharge containing suspended solids, oils, heavy metals, phenol, ammonia, cyanide etc. coming from entire plant area , thereby suggesting that the area is highly slushy, filled up with oil sludge, other heavy and hazardous metals. Most of the lagoon area on upstream of weir is found to be covered with elephantine grass and heavily silted up.

4. REVIEW OF LITERATURE:

If I have seen farther than others, it is because I have stood

On the shoulder of giants.

---- Sir Isaac Newton

Mercury resistant bacteria (MRB) are those bacteria that grow in presence of 10 ppm (mg/l) mercury ($HgCl_2$) in the nutrient agar medium (NA) (Nascimento et al., 2003). There are three important types of mercury which is the pure element, inorganic compounds such as mercuric nitrate and organic mercury compounds such as phenyl mercuric propionate. Elemental mercury is a liquid and gives off mercury vapor at room temperature. This vapor can be inhaled into the lungs and passed into the blood stream (Shen and Wang, 1995). Elemental mercury can also pass through the skin and into the blood stream and if swallowed usually passes out of the body without harm. Inorganic mercury compounds can also be inhaled and absorbed through the lungs, and may pass through the skin. Many inorganic mercury compounds are irritating or corrosive to the skin, eyes and mucus membranes as well. Organic mercury compounds can enter the body readily through all three routes-lungs, skin and stomach (Helm and Cox, 1975; Goldschmidt, 1954).

4.1. Mercury in the environment

Mercury, the only metal in liquid form at room temperature is the most toxic of the heavy metals (Dechwar et al., 2004) and the sixth most toxic chemical in the list of hazardous compounds (White et al., 2005) has been present in the environment for aeons. Erupted from the core of earth by volcanic activity it exists as mineral (mostly as cinnabar-HgS), as mercuric oxide, oxychloride, sulfate mineral (Kiyono and Pan Hau, 2006) or also as elemental mercury. It also exists as gas due to its high vapour pressure. In a biogeochemical cycle (Fig. 2) (Barkay et al., 2003) mercury is globally dispersed undergoing many physical and chemical transformations:

i. In the atmosphere elemental mercury is photo-oxidized to ionic mercury (Hg^{2+}).

ii. Rain precipitates the inorganic mercury on the surface of the earth, where carried out mainly by microorganisms in aquatic systems.

iii. It is reduced back to its elemental form or methylated.

iv. Elemental mercury evaporates into air where the cycle begins anew.

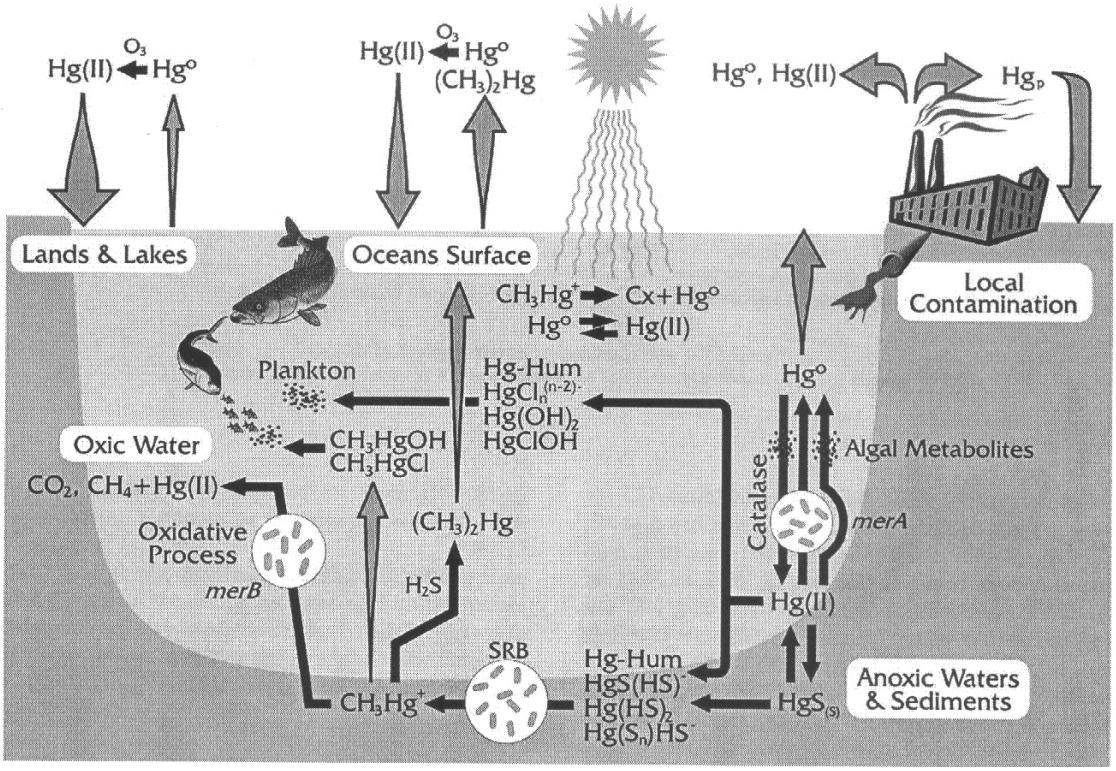


Figure 2. The biogeochemical cycle of mercury in the environment (Barkay et al., 2003).

Mercury is rapidly adsorbed onto sediments, which can serve as a source of mercury for years (Kornad, 1971; Matsumura et al., 1972; McDuffie et al., 1981). For this reason, most of the mercury available for cycling in freshwaters is present in bed sediments.

4.2. Toxicity of Mercury

Even small amounts of mercury are toxic for all organisms. Mercury binds to the sulfhydryl groups of enzymes and proteins, thereby inactivating vital cell functions (Dobler et al., 2000b).The most notable examples of environmental contamination with mercury occurred in Japan between 1953 and 1970 (Irukayama, 1966; Tsubaki, 1968). In

Minamata, between 1953and 1961, 121 fishermen and their families were stricken with a mysterious illness characterized by cerebellar ataxia, constriction of visual fields,and dysarthria. Of these 121 cases, a total of 46 deaths resulted. Additional cases of mercury-induced poisoning, termed "Minamata Disease," were seen in the coastal town of Niigata and in the riverside villages along the Agano River between 1965 and 1970 (Konrad, 1971). Six persons died and another forty-one were irreversibly poisoned. In both incidents, the disease broke out mainly among fishermen and their families, and also among other people who fished frequently and/or liked to eat locally caught aquatic produce. Characteristically, the patients in Minamata as well as in Niigata had eaten a great amount of fish and/or shellfish from contaminated waters.

4.2.1. Inorganic Mercury Compounds

The toxicity of heavy metals is a result of their binding to active sites of important enzyme systems in the cells and their binding to 1igands in the cell membrane thereby resulting in a variety of toxic effects (Passow et al., 1961). Inorganic mercury compounds concentrate in the kidney, liver and spleen. They are readily excreted, however, and do no damage unless the threshold tolerance level of the organ is exceeded (D'Itri, 1972). Prolonged exposure to inorganic mercury compounds is required for toxic symptoms to develop. The symptoms of inorganic mercury poisoning develop gradually. The first clear physical symptoms are numbness of the fingers and toes and then of the lips and tongue (D'Itri, 1972). Weakness, fatigue, anorexia, loss of weight and disturbances of gastrointestinal functions are associated with fully developed clinical forms of chronic poisoning (Friberg and Vostal, 1972). Late phases are characterized by mercurial tremor, psychic disturbances, and changes in personality (Friberg and Vostal, 1972). Prolonged exposure to high concentrations of inorganic mercury can result in death.

4.2.2. Organic Mercury Compounds

Organic mercury compounds are most toxic forms of mercury and can be divided into two categories: those in which the mercury atom is bonded to one organic radical and those in which it is bonded to two organic radicals.

The first type dissociates in water to yield the $R\text{-}Hg^{+}$ cation and the X^{-} anion, making it soluble in water. Mercury is covalently linked to a carbon atom in organic mercury compounds (Nordberg, 1976). Methyl mercury can be formed from mercuric ion by a variety of microorganisms, including anaerobes, facultative anaerobes, and aerobes. Thus, the potential for microbial methylation exists under both aerobic and anaerobic conditions. Most organic mercury compounds are rapidly excreted and therefore, pose no serious health problems (Jugo, 1979). However the short-chain alkyl mercury compounds, such as methyl mercury, are formed in aquatic environments via methylation of inorganic mercury. Methylmercury is among the most toxic of all mercury compounds (Cassidy and Furr, 1978; D'Itri, 1972).

The mercury-carbon bond in methyl mercury is extremely stable and the attachment of the alkyl radical increases lipid solubility. This facilitates penetration of the blood brain barrier and cell membranes (Felton et al., 1972). Nervous tissue tends to accumulate the greatest concentrations of methyl mercury (Chang and Hustman, 1972). Methyl mercury rapidly diffuses through the cell membrane and enters the cell where it is rapidly bound by sulfhydryl groups. Inside the cell, methyl mercury inhibits protein and RNA syntheses (Jugo, 1979). Methyl mercury concentrates in the body during a latent period during which no symptoms are observed, After threshold levels are exceeded, severe effects on the central nervous system may occur (D'Itri, 1972), Symptoms of methyl mercury poisoning include fatigue, headache, numbness of the extremities, blurred vision that can progress to blindness, and poor muscular coordination (Jugo, 1979).

4.3. Sources of Mercury

The four main natural processes that aid Hg emission are:

1. Degassing from geological mineral deposits

2. Emissions from volcanic activities
3. Photoreduction of divalent mercury in aquatic systems
4. Biological formation of elemental and methyl mercury

Although it is undisputed that mercury occurs naturally and toxic concentrations in some locations, mercury emissions owing to anthropogenic activities (mainly through chloralkali electrolysis and chlorine production), mining and fossil fuel combustion or waste incineration are immense, contributing substantially to the mercury pool participating in the biogeochemical cycle(Fig 2) (Komura et al., 1971). However, the concentrations of mercury in various compartments from natural and anthropogenic sources are highly variable.

4.4. Effects of Mercury Contamination on Microorganisms

A. Effects on microbial activities :

Few studies have been attempted to determine the effect of mercury contamination on other microbial activities. Pedersen and Sayler (Nordberg, 1976) found that $HgCl_2$ had no significant effects on methanogenesis. Research by Winfrey (unpublished) confirmed these resu1ts. The sediment environment may protect the methanogenic population from the toxic effects of mercury (Pederson and Sayler, 1981). Effects of mercury on other microbial activities have apparently not been investigated.

B. Ecology of mercury resistant bacteria :

Many bacteria possess a variety of resistance mechanisms to the toxic effects of mercury. Resistance depends on the strain, species, and genus of bacteria. Nelson and Colwell (Nelson and Colwell, 1975) showed that H_2S production is not an exclusive property of mercury resistant bacteria.

4.5. Bacterial Resistance to Mercury

As a response to toxic mercury compounds globally distributed by geological and anthropogenic activities, microbes have developed a surprising array of resistance mechanisms to overcome Hg toxicity (Pahan et al., 1990). However, some bacterial communities residing in the mercury contaminated areas can exchange mercury resistance genes between each other, because of continually exposure to the toxic levels of mercury. After the acquisition of resistance genes, those bacteria will be resistant to

mercury (Nascimento and Souza, 2003). An extensively studied resistance system based on clustered genes in an operon (i.e. mer), allows bacteria to detoxify Hg^{2+} into volatile mercury by enzymatic reduction (Deckwer et al., 2004; White et al., 2005; Kiyono and Pan Hau, 2006). It appears that bacterial resistance to mercury is an ancient mechanism, probably acquired even before anthropogenic usage of mercury. Since the same biotransformation that constitute the Hg biogeochemical cycle can take place inside the human body, understanding its external transformations and transport processes will help in figuring out which of these processes can exacerbate or ameliorate Hg toxicity in humans (Barkay et al., 2003).

4.5.1. Biochemical Basis and Molecular Basis of Bacterial Mercury Resistance

i. Formation of insoluble HgS

In the presence of hydrogen sulfide, mercuric ions (Hg^{2+}) spontaneously precipitate as mercuric sulfide (HgS) (Furukaura et al., 1969). Under anaerobic conditions, the formation of mercuric sulfide effectively reduced availability of mercuric ion for biological conversions. In the presence of oxygen, mercuric sulfide may be converted to methyl mercury by bacteria, however, this occurs at a rate 100-1000 times slower than mercuric ion methylation (Fagerstrom and Jernelov, 1971). Therefore, the presence of sulfide reducing bacteria prevents methyl mercury ($(CH_3)_2Hg$) and mercuric sulfide in the presence of hydrogen sulfide. Mercuric ion may also be reduced to the volatile elemental mercury by resistant bacteria. This reaction results in the release of mercury from aquatic systems (Colwell et al., 1976).

Mercury volatilization might be expected to occur readily than methylation due to the large numbers of bacteria capable of carrying out this reaction in aquatic sediments (Colwell et al., 1976) and the kinetics of volatilization in bacterial cultures compared to methylation (Anne Summers, personal communication).

ii. Enzymatic reduction Hg^{2+} to Hg^0 and volatilization

The biochemical basis of resistance to inorganic mercury compounds such as $HgCl_2$ appears to be quite similar in several different species (Canovas et al., 2003). It involves the reduction of Hg^{2+} to volatile Hg^0 by an inducible enzyme, mercuric reductase. This reductase is a flavoprotein, which catalyzes the NADPH-dependent reduction of Hg^{2+} to Hg^0. Since mercury has such a high vapor pressure, it volatilizes and the bacterial environment is left mercury free. This mercuric reductase is found intracellularly (Furukawa and Tonomura, 1972; Summers1972; Schottel, 1978).

As a response to toxic mercury compounds globally distributed by geological and anthropogenic activities, microorganisms have developed a surprising array of resistance systems to overcome the poisonous environment (Canstein et al., 1999). An extensively studied resistance system, based on clustered genes in an operon (*mer* operon), allows bacteria to detoxify Hg^{2+} into volatile metallic mercury by enzymatic reduction (Komura and Izaki, 1971; Summers, 1986; Misra, 1992; Silver, 1996; Osborn et al., 1997). Mercury-resistance determinants have been found in a wide range of Gram-negative and Gram-positive bacteria isolated from different environments. They vary in the number and identity of genes involved and are encoded by *mer* operons, usually located on plasmids (Summers and Silver, 1972; Brown et al., 1986; Griffin et al., 1987; Radstrom et al., 1994) and chromosomes (Wang et al., 1987; Inoue et al., 1991); they are often components of transposons (Misra et al., 1984; Kholodii et al., 1993) and integrons (Liebert et al., 1999).Two main *mer* determinant types have been described: narrow-spectrum *mer* determinants confer resistance to inorganic mercury salts only, whereas broad-spectrum *mer* determinants confer resistance to organomercurials such as methyl mercury and phenyl mercury, as well as to inorganic mercury salts (Misra, 1992; Silver and Phung, 1996; Bogdanova et al., 1998). The functions of *mer* operon are as follows:

a. Transport of Hg^{2+} into the cell

b. Enzymatic NADPH dependent conversion of the ionic mercury into relatively less toxic elemental mercury (Hg^0)
c. Regulation of the functional genes
d. Cleavage of mercury from the organic residue and the resistance is termed as "Broad spectrum"

The genes involved in *mer* operon are shown in Fig 3:

a) *mer T*, *mer P* (Transport)
b) *mer A* (Mercury reduction)
c) *mer B* (Cleavage of mercury from organic residue)
d) *mer R* and *mer D* (regulation)
e) *mer C* and *mer F* (Membrane proteins, conferring transport functions)
f) *mer G* (resistance to phenyl mercury)

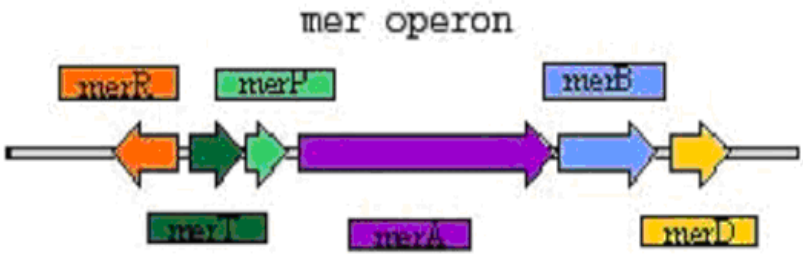


Figure 3. The *mer* operon including the regulators (*merR* and *merD*), transporters (*merP* and *merT*), mercuric reductase (*merA*) and organomercurial lyase (*merB*).

Different mer genes in mer operon play different roles. The functions of these genes are as follows:

1. ***mer R*:** Metalloregulatory DNA binding protein that acts as a repressor of both its own and structural gene transcription in the absence of Hg (II). In addition it acts as a positive effector of structural gene transcription when Hg (II) is present.

2. ***mer B*:** Organomercury lyase, catalyzes the protonolytic fragmentation of organomercurials to the parent hydrocarbon and Hg(II) by S_E2 mechanism as shown in Fig 4.
3. ***mer A*:** Mercuric ion reductase, is an FAD containing and redox active disulfide containing enzyme with homology to glutathione reductase.This enzyme reduces Hg^{2+} compounds to the metallic mercury Hg ^{0} which is obviously less toxic to them (Deckwer et al., 2004). It has the unique capacity to reduce Hg(II) to Hg(0) and thereby complete the detoxification scheme as shown in Fig 4.

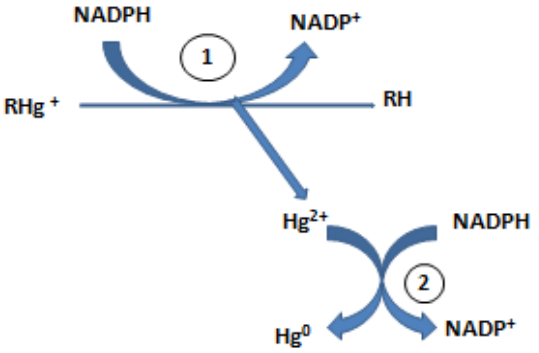


Figure 4. Detoxification of mercury by organomercurial lyase (1) and mercuric reductase (2) enzymes. (Adapted from Summers and Silver, 1978)

Based on a comparison with other bacterial periplasmic binding, protein-dependent transport systems, it has been proposed that Hg^{2+} diffuses across the outer membrane (Brown, 1985). Mercuric ions are transported outside the cell by a series of transporter proteins. This mechanism involves the binding of Hg^{2+} by a pair of cysteine residues on the *merP* protein located in the periplasm as shown in Fig 4 (Chang et al., 1993). Hg^{2+} is then transferred to a pair of cysteine residues on *merT*, a cytoplasmic membrane protein, and finally to a cysteine pair at the active site of MerA (mercuric reductase) (Hamlett et al., 1992). Next, Hg^{2+} is reduced to Hg^0 in an

NADPH-dependent reaction. The non-toxic Hg^0 is then released into the cytoplasm and volatilizes from the cell.

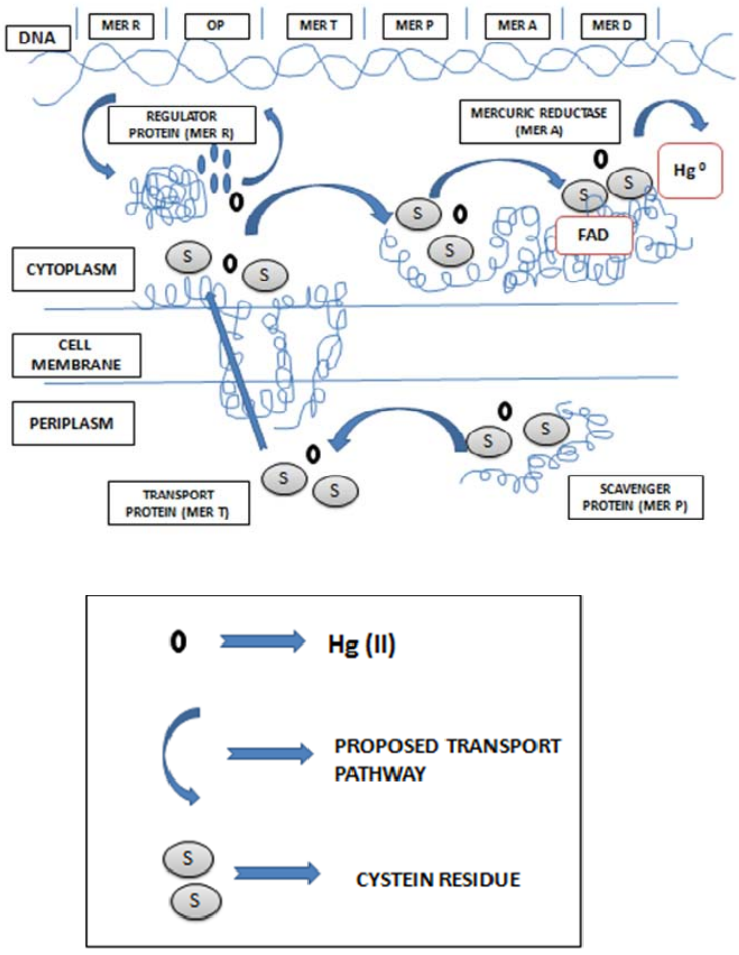


Figure 5. Proposed scheme for sequestration of Hg (II) ions in the periplasm and their sequential passage to the active site of mercuric reductase (*merA*) by a brigade mechanism involving the paired cysteine residues of *merP*, *merT*, *merA*. (Adapted from Silvet and Kinscherf, 1982)

5. MATERIALS AND METHODS:

Theory guides, experiments decides

---------- Anonymous

5.1. Sample Collection

Four different sites were selected in the inlet lagoon of Rourkela Steel Plant, Orissa. Representative sediment samples were collected in sterile plastic bags during the month of January 2011 (Fig 6). Samples then transported aseptically and processed immediately in the laboratory for enumeration of viable cell count.

Figure 6. Collection of sediment samples

5.2. Enumeration of Viable Cell Count

Enumerations of mercury resistant heterotrophic bacteria were conducted using plate technique. Varying concentrations (5ppm, 10ppm) of mercuric ion (as $HgCl_2$) were added

to Nutrient Agar (NA) from a 1000ppm stock ($HgCl_2$). All samples were serially diluted in autoclaved distilled water up to 10^5.

Aliquots of 0.1ml from each dilution was spread on Nutrient Agar (NA) media plates supplemented with 10ppm, 5ppm of $HgCl_2$ and without $HgCl_2$. The plates were incubated at 37^0C for 24 hours. After incubation period, the appeared colonies on both NA containing Hg (II) and without Hg (II) were enumerated using total viable plate count method (Prescott and Harley, 2002) and expressed as Colony Forming Units (CFU)/g.

C.F.U/mg original sample = Number of Colonies/plate * (1/ml aliquot plated) * dilution factor

5.3. Isolation and Identification of Mercury Resistant Bacteria

Isolation of mercury resistant bacteria was done by directly plating on Nutrient Agar (NA) containing mercury (II). In directly method after enumerating the number of bacteria the appeared colonies on appropriate plates containing Hg (II) were purified and streaked onto Nutrient Agar (NA) plates which were further incubated for 24 hours. The pure cultures of isolated strains were preserved in Nutrient Agar (NA) supplemented with 10ppm $HgCl_2$ slants in vials under refrigerated (4^0C) conditions and coded as 1S3 to 4S4 for further uses. Then the appeared colonies were identified with gram staining. The gram negative isolates were identified by standard biochemical tests (Collins and Lyne, 1970; Hansen and sorheim, 1991) as per the requirements of bacterial identification software Abis 6 online (Byrant, 2004) and Bergey's manual of determination bacteriology.

$$\text{Percentage (\%) Mercury Resistant Bacteria (MRB)} = \frac{\text{Mercury resistant bacteria}}{\text{Total heterotrophic bacteria}} * 100$$

5.4. Gram's Staining

To study the gram's stain (crystal violet) i.e. Gram (+ve) or Gram (–ve) characters of the isolates, diluted suspensions of the bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times. The slides, were flooded with crystal violet

solution for one minute, washed with water and flooded with Gram's iodine for one minute.

The slide were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off solution. The slides were washed with water and counter stained with safranin stain for about 30 second and washed with water. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter. Cells were then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

5.5. Colony Morphology

Size, shape, color, elevation and margins of the bacterial colonies were observed for 24 hours incubated cultures, on the Nutrient agar (NA) media plates supplemented with 10ppm $HgCl_2$.

5.6. Cell Morphology

The shape and size of the cells were observed by the Microscope. Motility test can be used to check for the ability of bacteria to migrate away from a line of inoculation thanks to physical features like flagella. To perform this test, the bacterial sample is inoculated into SIM or motility media (mannitol) using a needle. Simply stab the media in as straight a line as possible and withdraw the needle very carefully to avoid destroying the straight line. After incubating the sample for 24-48 hours observations can be made. Observe if the bacteria have migrated away from the original line of inoculation.

- If migration away from the line of inoculation is evident then the test organism is motile (positive test).
- Lack of migration away from the line of inoculation indicates a lack of motility (negative test result).

5.7. Determination of Minimum Inhibitory Concentration (MIC)

It means the lowest concentration of metal that completely prevented bacterium growth (De and Ramaiah, 2007; Gupta et al., 2005). MIC test was done by Broth Tube Dilution method (Fig 7) which is as follows:

1. Sterile capped test tubes were taken.
2. 2.0ml of 200ppm $HgCl_2$ added into 1st test tube.
3. 1.0ml of sterile Mueller Hinton Broth (MHB) added in test tube 2nd, 3rd, 4th,5 th,6 th,7 th,8 th,9 th,10 th,11 th,12th respectively.
4. 1ml of the mixture was transferred from 1st test tube to the 2nd tube and this procedure was repeated till the 11th test tube.
5. From the 11th test tube 1ml of the media discarded that means 11th test tube contains 1ml MHB media and 1ml of 200 ppm $HgCl_2$.
6. 1ml of diluted culture suspension added to each of the tubes.
7. Tubes were incubated at 37^0C for 24 hour (+/- 2 hour).
8. OD was taken at 630nm using Spectrophotometer (ELICO BL200B10 Spectrophotometer).

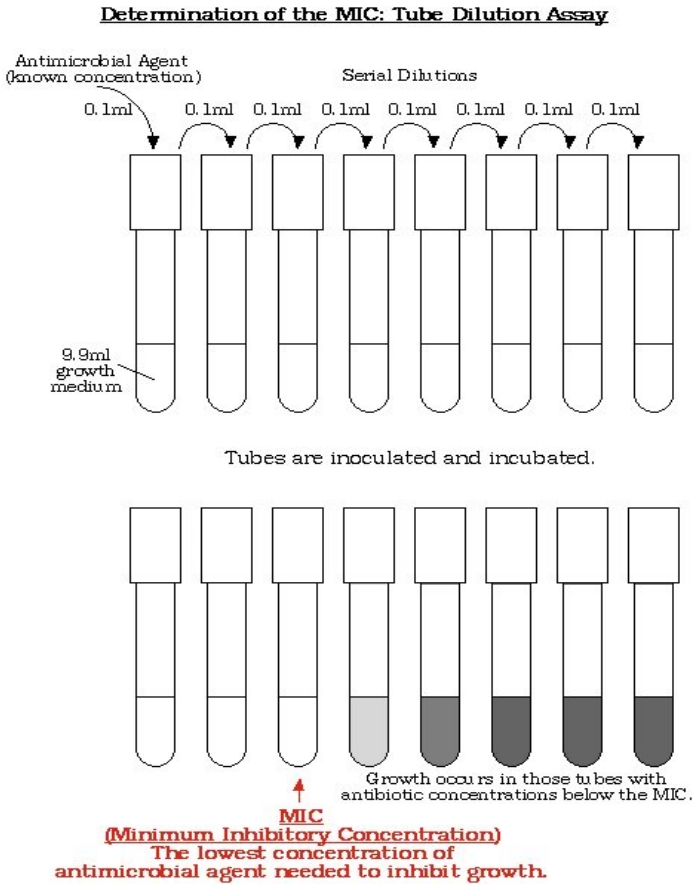


Figure 7. MIC Broth Dilution Tube Method

5.8. Growth Characterization on Different Media

The growth behavior of strains was studied on different media, i.e. MacConkey's media (Hi media, Mumbai).

5.9. Physico-Chemical Parameters

Physico-chemical parameters like temperature, pH, salinity, metal tolerance tests were analyzed.

i. Estimation of pH tolerance of bacterial isolates :

The pH tolerance test was conducted to study the cardinal pH of the mercury resistant bacteria (MRB) (Buchanan and Gibbons, 1977; Holt et al., 1994). The procedure for estimation of pH tolerance is as follows:

1. 3ml of the medium was taken in different test tubes and the pH was adjusted from 6-10 respectively with the help of 1N HCl, 1N NaOH.

2. 100µl of the overnight culture (Luria Bertani, LB) was dispensed into the test tubes and incubated at 37^0C for 24 hours.
3. OD was taken at 630nm using Spectrophotometer (ELICO BL200B10).

ii. Effect of temperature on bacterial growth

The temperature is an important factor to which bacteria show a wide pattern on growth behavior. The procedure for study the effect of temperature on bacterial growth is as follows:

1. 3ml of the medium was taken in different test tubes.
2. 100µl of the overnight culture (Luria Bertani, LB) was dispensed into the test tubes.
3. The test tubes were incubated at 3 different temperatures i.e. 37°C, 25°C, 45°C for 24 hours respectively.
4. OD was taken at 630nm using Spectrophotometer (ELICO BL200B10 Spectrophotometer).

iii. Effect of salinity on bacterial growth

In the marine and estuary sediments, the contribution of methyl mercury in the concentration of total mercury is ~ 0.5%, whereas in the fresh water sediments it usually reaches 1~ 1.5% (Hamasaki et al., 1995; Kannan and Falandysz, 1998). One of the reasons for a smaller contribution of methyl mercury in the marine and estuary sediments is that the chlorine ions affect the processes of mercurymethylation and demethylation (Guimaraes et al., 1998; Compeau and Barthar, 1983). The rate of mercurymethylation decreases with increasing concentration of salt, which is most probably a result of the inhibitory effect of chlorine complexes. The procedure for study the effect of salinity on bacterial growth is as follows:

1. 3ml of the medium was taken in different test tubes and the salinity was adjusted as 0.2, 0.4, 0.6, 0.8, and 1.0 respectively with the help of 1N NaCl.

2. 100µl of the overnight culture (Luria Bertani, LB) was dispensed into the test tubes and incubated at 37^0C for 24 hours.
3. OD was taken at 630nm using Spectrophotometer (ELICO BL200B10 Spectrophotometer).

iv. Resistance to metals

For checking metal resistance of the isolates, the media were supplemented with different concentrations of metal salts like $CuSO_4.5H_2O$, $CdCl_2.H_2O$, Ferrous sulphate, Zinc sulphate (Buchanan and Gibbons, 1977; Holt et al., 1994). The procedure for study the resistance of isolates to metals is as follows:

1. Stock solutions of different metal salts were prepared.
2. 3ml of the medium was taken in different test tubes at different concentrations like 10ppm, 20ppm, 40ppm, 80ppm respectively.
3. 0.2µl, 0.4µl, 0.8µl, 1.6µl of Stock solution of metal salts were added to 10ppm, 20ppm, 40ppm, 80ppm concentrations.
4. 100µl of the overnight culture (Luria Bertani, LB) was dispensed into the test tubes and incubated at 37^0C for 24 hours.
5. OD was taken at 630nm using Spectrophotometer (ELICO BL200B10 Spectrophotometer).

5.10 Biochemical Characterization

Himedia Rapid Biochemical Identification kit, *Enterobacteriaceae* Identification Kit [KB003 Hi25®] was used in the present study as KB003 is the comprehensive test system used for identification of gram negative *Enterobacteriaceae* species. Single well isolated colony was picked up and inoculated in 10 ml NA broth and incubated at 37ºC for 24 hours. Oxidase test was performed on organism to be tested to differentiate Enterobacteriaceae from other gram negative rods using the Oxidase disc provided with the kit. Kit was opened aseptically and sealing tape was peeled off. Each well was inoculated with 50 µl of the above inoculums by surface

inoculation method and kept for inoculation at 35- 37°C for 18-24 hours. At the end of the incubation period, a series of reagents were added in designated wells as per manufacturer's specifications to carry out different biochemical tests.

For the ultimate characterization of isolated strains the following biochemical tests were performed that are being discussed as below:

- **Triple Sugar Iron (TSI) Test**

  Principle: To detect utilization of glucose, sucrose, and lactose by bacteria producing acids. Acid (low pH) is detected by indicator phenol red which changes from yellow in acidic to pink alkaline condition (Holt et al., 1994) (Fig 8). Also production of H_2S is detected if blackening is observed.

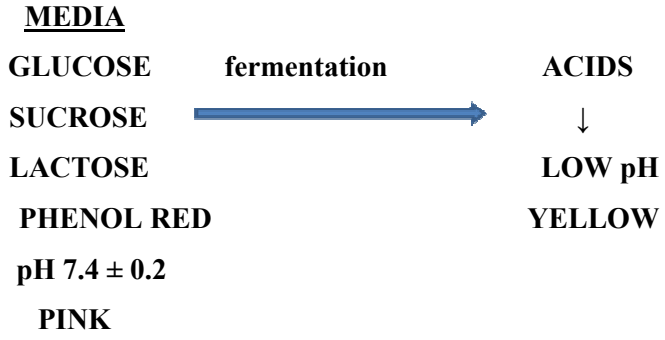


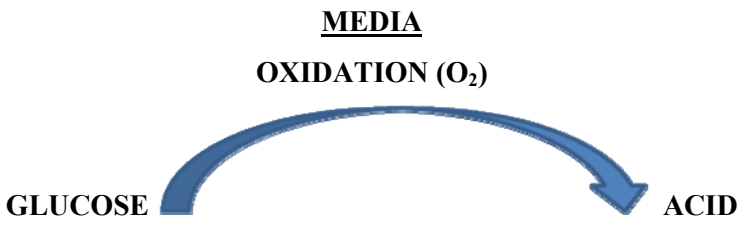
Figure 8. Mechanism of Triple Sugar Iron test

- ✓ **Procedure :**
  a) TSI agar media is prepared from readymade TSI agar, sterilized, and slants and butt are prepared in test tubes.
  b) Bacterial inoculation is done by stabing into butt and streaking on slant.
  c) Tubes are incubated at 37°C for 24 hours.

- Yellow butt, pink slant: Acid butt, alkaline slant is formed. Here only glucose has been fermented anaerobically. So acidic butt. No gas or H_2S production. No other sugar has been utilized. Since glucose is very less (0.1 %), after its utilization oxidative deamination of peptone occurs and thus NH_3 is produced giving pink colour alkaline slant (i.e. aerobic condition).
- Yellow butt and Yellow slant: Acid butt and slant is formed.
    a) **No H_2S and gas**: Here glucose is fermented. Lactose and/or sucrose is fermented.
    b) **H_2S produced**: Blackening along the line of incubation with above condition. It is due to the reduction of sulphite to sulphide (as $NaHSO_4$ is present in media). Further sulphate present in the medium will be converted to respective sulphide and black colour occurs.

➢ **Oxidation Fermentation Test (O/F Test)**

Principle: This test is performed to test whether glucose utilization in bacteria takes place in presence of oxygen (i.e. oxidatively or aerobically) or in absence of it (i.e. fermentatively anaerobically) (Fig 9).A bacteria able to ferment glucose must be able to oxidize it. But reverse may not be true. If glucose is utilized in either way, acid is produced changing the colour from purple (due to bromocresol purple used in media) to yellow by lowering pH (Holt et al., 1994; Anonymous, 2000).



BROMOCRESOL PURPLE pH 7.4 **FERMENTATION (WITHOUT OXYGEN) PURPLE** **LOW pH YELLOW**

Figure 9. Mechanism of Oxidation Fermentation Test (O/F Test)

✓ **PROCEDURE**

a) Hugh-Leifson Glucose Broth (HLGB) with glucose, bromocresol purple, as main component and pH 7.4 is prepared. Less agar is used to get semisolid media to facilitate stab. Then distributed into test tubes and sterilized.

b) Inoculation is done by stabbing of loop.

c) Over one set of tubes paraffin is poured to give anaerobic condition and then incubated for 24 hours and the other set incubated.

- Colour changed from purple to yellow in both tubes: fermentative.
- Colour changed only in tubes without paraffin: oxidative.
- No colour change in any tube: microorganism is inert to the media.

5.11. Biochemical Identification Kit

Commercially available systems reduce the need for preparing a variety of test media and reagents and the time required for interpretation of results, thereby making the identification of various bacterial species more plausible in the routine laboratory. Himedia Rapid Biochemical Identification kit, *Enterobacteriaceae* Identification Kit [KB003 Hi25®] and Motility Test kit (KBM001 to KBM003) are some of the methods used worldwide. HiMedia provides a range of Biochemical Identification test kit (KB001 to KB012) involving single step procedure of inoculation which leads to final identification of test organism being studied. Each

Biochemical Identification test kit is a standardized colorimetric identification system utilizing conventional biochemical tests and carbohydrate utilization tests. The tests are based on the principle of pH change and substrate utilization. On incubation organisms undergo metabolic changes which are indicated by a colour change in the media that is either interpreted visually or after addition of a reagent.

6. RESULTS AND DISCUSSION:

Facts are not science: as the dictionary is not Literature

- - - - - Martin H Fischer

6.1. Bacterial Enumeration

Total viable counts ranged from $20*10^4$ (CFU/mg) in site 1 sample to $4.5*10^5$ (CFU/mg) in site 3 sample as shown in Table 1. The frequencies of resistance to mercury varied from 42% in site 1 sample to 83.93% in site 4 sample as shown in Table 1. Comparison of Total Heterotrophic Bacteria (THB) and Mercury Resistant Bacteria (MRB) has been shown in Fig 10. A percentage of mercury

resistant bacteria in sediments at site 1,2,3,4 are 42%, 26.32%, 67.56%, 83.93% respectively.

Table 1. Total Heterotrophic Bacteria

Dilution Factor	**Sample number**	***THB (CFU/g)**	***MRB(CFU/g)**		**%age Mercury Resistant Bacteria (MRB)**
			5ppm	**10ppm**	
10^{-2}	S1	$20*10^4$	$16.4*10^4$	$8.4*10^4$	42%
10^{-2}	S2	$19*10^4$	$8.8*10^4$	$5.0*10^4$	26.32%
10^{-2}	S3	$4.5*10^5$	$3.4*10^5$	$3.04*10^5$	67.56%
10^{-2}	S4	$11.2*10^4$	$9.8*10^4$	$9.4*10^4$	83.93%

*THB: Total Heterotrophic Bacteria

*MRB: Mercury Resistant Bacteria

*%age: Percentage

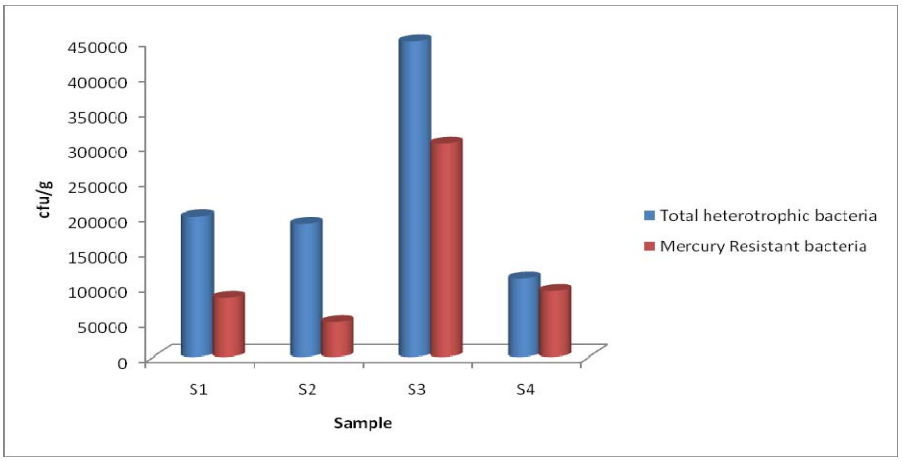


Figure 10. Comparison of Total Heterotrophic Bacteria and Mercury Resistant Bacteria in sediment sample

6.2. Minimal Inhibitory Concentration Test (MIC)

The minimal inhibitory concentration test (MIC) of $HgCl_2$ for 20 strains is shown in Table 2 and Fig 11.

Table 2. MIC Results of Bacterial Isolates

Sample Name	MIC (ppm)
S48	12.5
4S4	25
3S3	25
S1 2'	12.5
S3 1'	1.562
3S 1	25
S3 4'	3.125
S2 2'	12.5
1S3	6.25
4S3	100
2S1	25
S4 5	50
2S2	12.5
S3 2'	3.125
4S1	50
S2 3'	6.25
S1 2"	25
S1 3'	25
S4 9	12.5

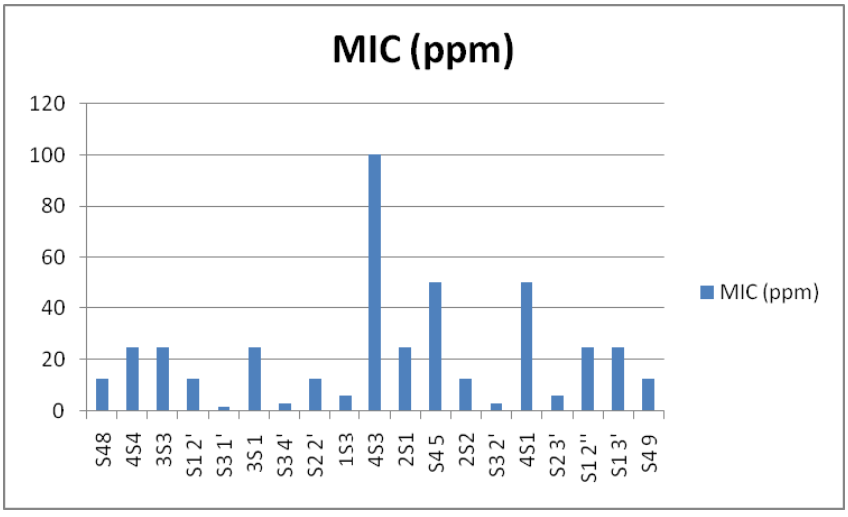


Fig 11. MIC results for bacterial isolates

Out of 20 bacterial isolates only 3 bacterial isolates shows highest MIC. These are S4 5, 4S3, 4S1 respectively.

6.3. Colony Morphology

The observed colony morphological characteristics pertaining to color, shape, and elevation are collectively displayed at Table 3.

Table 3. Colony Morphology of Isolated Strains

Strain Name	Color	Shape	Elevation
S48	Translucent	Irregular	Flat
4S4	White	Irregular	Flat
3S3	Off White	Circular	Flat
S1 2’	White	Circular	Raised
3S1	Translucent	Circular	Convex
S3 1’	Translucent	Circular Shiny	Convex
S3 4’	Yellowish	Irregular	Flat
S2 2’	Reddish	Circular	Flat
1S3	Creamy Off White	Irregular	Convex
4S3	Brown	Circular	Convex
2S1	Light Brown	Irregular	Convex

6.4. Growth Characterization on MacConkey’s Media

Growth behavior of 3 bacterial isolates viz. S4 5, 4S3, 4S1 is shown in Table 4. MacConkey is very important media as it supports only the growth of gram negative bacteria. It also differentiates lactose fermenters from non-lactose fermenters.

Table 4. Growth behavior of 3 bacterial isolates on MacConkey’s Media:

Strains Name	MacConkey’s Media
S4 5	White colony
4S1	White colony
4S3	White colony

6.5. Biochemical Characterization

Different biochemical tests were performed to characterize the mercury resistant bacterial strains and observations are collectively given in Table 5 and Table 6 respectively.

Table 5. Biochemical Test Results

KB003 ---- STRIP 1

Test	**4S3**	**S4 5**	**4S1**
ONPG	–	–	+
Lysine Utilization	–	–	+
Ornithine Utilization	–	–	–
Urease	–	–	–
Phenylalanine Deamination	–	–	–
Nitrate Reduction	–	–	–
Hydrogen Sulphide Production	–	–	–
Citrate Utilization	+	+	+
Voges Proskauer's	–	–	–
Methyl Red	+	+	+
Indole	–	–	–
Malonate	+	+	+

KB003 ---- STRIP 2

Test	**4S3**	**S4 5**	**4S1**
Esculin Hydrolysis	+	+	+
Arabinose	–	–	+
Xylose	–	–	–
Adonitol	–	–	–
Rhamnose	–	–	–
Cellobiose	–	–	–
Melibiose	–	–	+
Saccharose	+	+	+
Raffinose	–	–	+
Trehalose	+	+	+
Glucose	+	+	+
Lactose	–	–	–
Oxidase	–	+	–

KB009 ---- CARBOHYDRATE UTILIZATION

Test	4S3	S4 5	4S1
PART A	–	–	+
Lactose	–	–	+
Xylose	+	+	+
Maltose	+	+	+
Fructose	+	+	+
Dextrose	+	+	+
Galactose	–	–	+
Raffinose	–	–	+
Trehalose	+	+	+
Melibiose	–	–	+
Sucrose	+	+	+
L-Arabinose	–	–	–
Mannose	–	+	–
PART B			
Inulin	–	–	+

Sodium Gluconate	–	–	–

Strain Name	Motility Test	O/F Test	TSI Test
S4 5	+	Aerobic	+
4S3	+	Aerobic	+/-
4S1	+	Aerobic	+

Glycerol	–	+	+
Salicin	+	–	–
Dulcitol	–	–	–
Inositol	–	–	–
Sorbitol	–	–	–
Mannitol	–	–	+
Adonitol	–	–	–
Arabitol	–	–	–
Erythritol	–	–	–
Alpha-Methyl-D-Glucoside	–	–	–
PART C			
Rhamnose	–	–	–
Cellobiose	–	–	–
Melezitose	–	–	–
Alpha-Methyl-D Glucoside	–	–	–
Xylitol	–	–	+
ONPG	–	–	+
Esculin Hydrolysis	+	+	+
D-Arabinose	–	–	–
Citrate Utilization	+	+	+
Malonate Utilization	–	+	–
Sorbose	–	–	–
Control	–	–	–

Table 6. Biochemical Test Results using media:

+: Positive

- : Negative

S4 5 was aerobic, Urease negative, MR positive, VP negative, motile and gives no blackening in H_2S media. According to observed characters, S4 5 belongs to *Streptococcaceae* family. 4S3 was aerobic, Urease negative, MR positive, VP negative, reduce nitrate, motile and gives no blackening in H_2S media. According to observed characters, 4S3 belongs to *Enterobacteriaceae* family. 4S1 was aerobic, Urease negative, MR positive, VP negative, ONPG positive, motile and gives no blackening in H_2S media. According to observed characters, 4S1 belongs to *Enterococcus* family.

6.6. CELL MORPHOLOGY

Cell morphology of strains was studied and observations are described as in Table 7.

TABLE 7. Cell Morphology of Isolated Strains.

Strain Name	**Colour**	**Gram Staining**	**Shape**
4S3	Pink	-ve	Rods
S4 5	Purple	+ve	Cocci
4S1	Purple	+ve	Cocci
S4 9	Pink	-ve	Cocci
3S1	Pink	-ve	Cocci
1S2	Purple	+ve	Bacillus

2S2	Purple	+ve	Cocci
S3 4	Pink	-ve	Cocci
3S2	Pink	-ve	Cocci
S2 1	Pink	-ve	Cocci
1S4	Purple	+ve	Bacillus
S2 3	Pink	-ve	Cocci
S2 2’	Purple	+ve	Cocci
1S2	Purple	+ve	Bacillus
2S1	Purple	+ve	Bacillus
4S3	Pink	-ve	Cocci
4S1	Pink	-ve	Cocci
3S1	Pink	-ve	Bacilli
S3 1’	Pink	-ve	Cocci

After performing different biochemical tests and gram staining, results obtained were put into Abis 6 online software and it is confirmed that S4 5 is *Streptococcus canis* (87%), 4S3 is *Pragia fontium* (81%), 4S1 is *Enterococcus saccharolyticus* (75%) respectively.

6.7. Growth Characterization at Different pH’s

Effect of pH was observed by growing the bacterial strains on Luria Bertani (LB). Optical density (Absorbance) was taken at 630nm wavelength. The trend of pH is recorded in Fig 12. The pH of the environment affects bacterial growth. It is cleared from Fig 12 that S4 5 showed most appropriate growth at pH 7. Hence, it preferred neutral pH more than the alkaline pH where growth was less. 4S3 showed the same trend as in case of S4 5 but the growth was drastically reduced at alkaline pH (pH 10) . 4S1 grew the best at acidic pH (pH 6). Overall, mercury resistant bacteria (S4 5and 4S1) can tolerate acidic pH as well as alkaline pH while 4S3 growth was reduced as pH go on becoming alkaline.

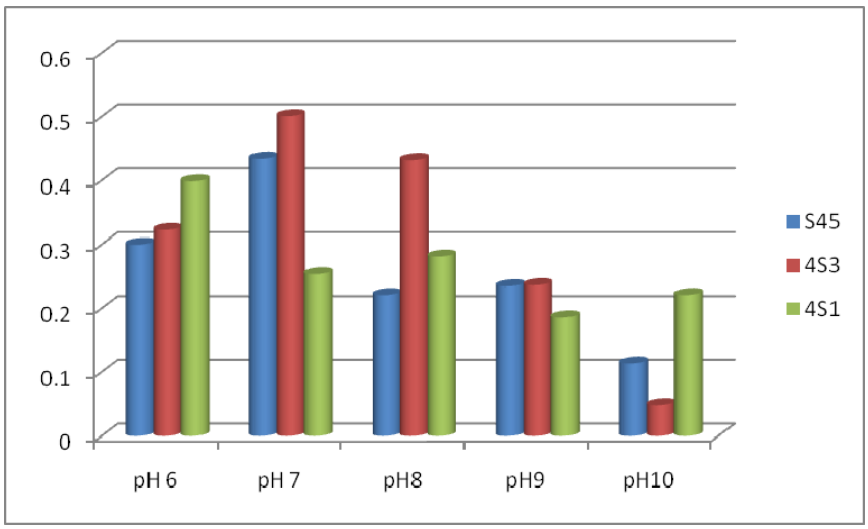


Fig 12. Effect of pH on bacterial growth

6.8. Growth Characterization at Different Temperatures

All strains were incubated at 25°C, 37°C, and 45°C using Luria Bertani (LB) for 24 hours. The growth was determined at the different temperatures at 630nm wavelength and the records recorded as shown in Fig 13.

The temperature is an important factor to which bacteria show a wide pattern on growth behavior. It is cleared from graph and Table 4S3 and 4S1 showed maximum growth at 25°C and 45°C respectively and growth was largely reduced at 37°C and 25°C. S4 5 showed maximum growth at 45°C while minimum growth at 25°C. Over all, in all strains the optimum temperature was found to be 45°C and 25°C respectively.

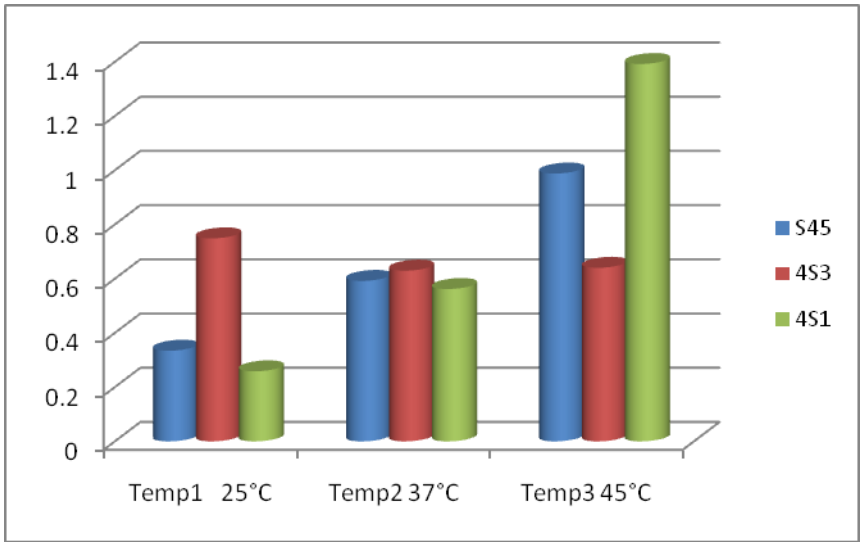


Fig 13. Effect of temperature on bacterial growth

6.9. Effect of Salinity on Bacterial Growth

The salt concentration in an environment is the major contributor to the osmotic effect of ions on growth. Bacteria require ions that are provided by salts and typically moderate salt concentrations. High salt or high sugar in the environment leads to loss of water from cells and, ultimately, to the death.

Some bacteria require an astonishingly high level of salt to begin growth, whereas other bacteria would be immediately killed in high levels of salt. The results were shown in Fig 14. Three strains viz. S4 5, 4S3, and 4S1 showed a moderate growth in different concentrations of NaCl.

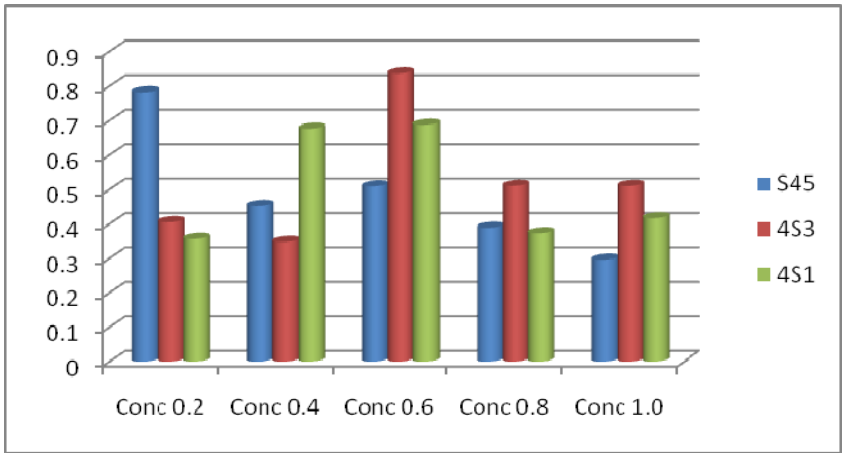


Fig 14. Effect of salinity on bacterial growth

6.10. Resistance of bacterial isolates to Metals

Resistance of bacterial strains to different metals is shown in Fig 15, fig 16, Fig 17 and Fig 18 respectively.

- **Zinc Metal :** S4 5 strain showed tolerance in all concentrations (10ppm,20ppm, 40ppm, 80ppm) but showed highest tolerance in 40ppm concentration of $ZnSO_4$. 4S3 showed highest tolerance in 20ppm concentration of $ZnSO_4$. 4S1 showed highest tolerance in 10ppm concentration of $ZnSO_4$ but showed weak growth with increased concentrations.

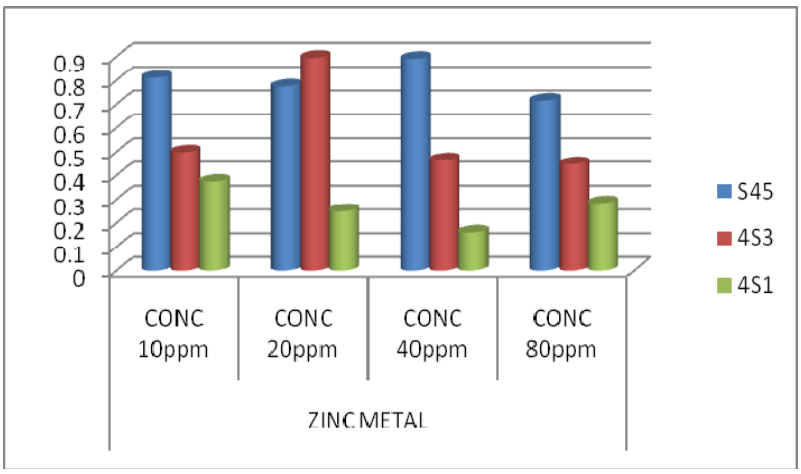


Fig 15. Resistance bacterial isolates to Zinc metal

- **Copper Metal :** S4 5 strain showed tolerance in all concentrations (10ppm,20ppm, 40ppm, 80ppm) but showed highest tolerance in 40ppm concentration of $CuSO_4.5H_2O$. 4S3 showed highest tolerance in 10ppm concentration of $CuSO_4.5H_2O$. 4S1 showed highest tolerance in 20ppm concentration of $CuSO_4.5H_2O$.

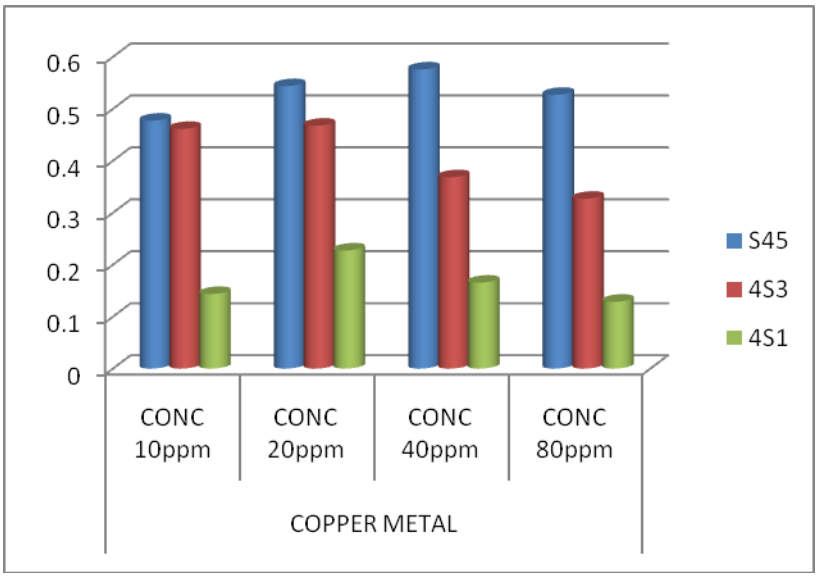


Fig 16. Resistance of bacterial isolates to Copper metal

- **Cadmium Metal :** S4 5 strain showed tolerance in all concentrations (10ppm,20ppm, 40ppm, 80ppm) but showed highest tolerance in 10ppm concentration of $CdCl_2.H_2O$. 4S3 showed highest tolerance in 10ppm concentration of $CdCl_2.H_2O$. 4S1 showed highest tolerance in 20ppm concentration of $CdCl_2.H_2O$ but showed weak growth with increased concentrations.

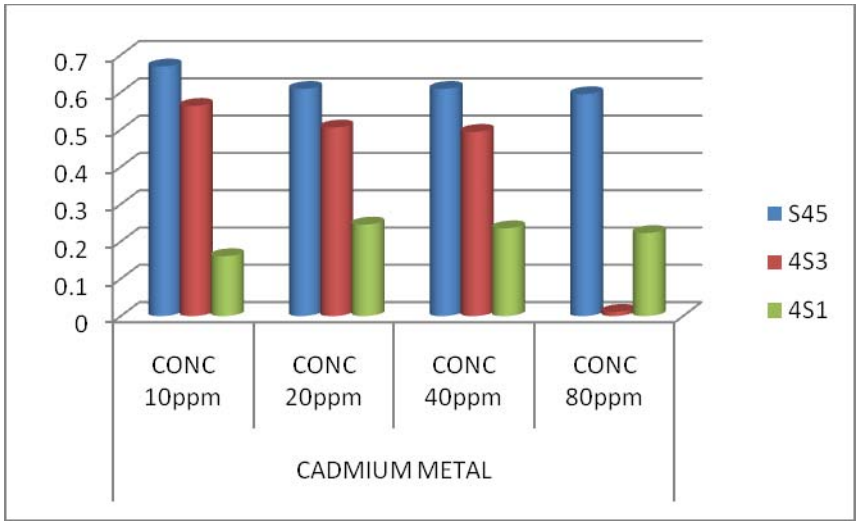


Fig 17. Resistance of bacterial isolates to cadmium metal

- **Iron Metal :** S4 5 strain showed tolerance in all concentrations (10ppm,20ppm, 40ppm, 80ppm) but showed highest tolerance in 10ppm concentration of $FeSO_4$. 4S3 showed highest tolerance in 20ppm concentration of $FeSO_4$. 4S1 showed highest tolerance in 40ppm concentration of $FeSO_4$ but showed weak growth with increased concentrations.

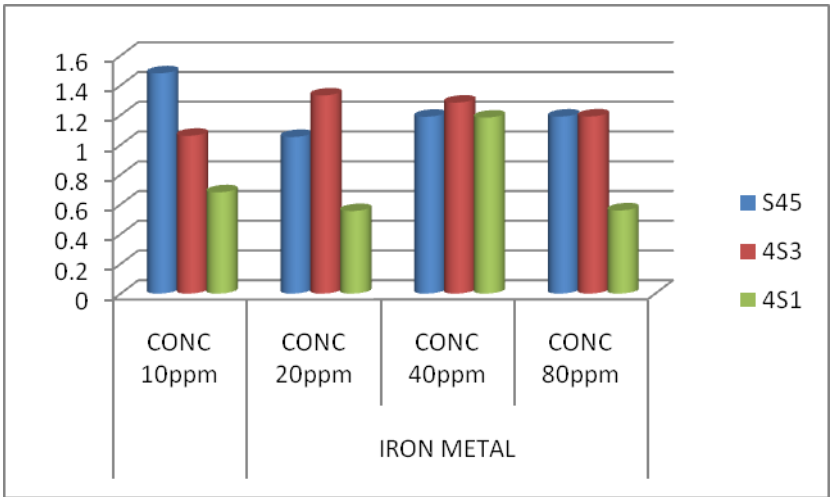


Fig 18. Resistance of bacterial isolates to Iron metal

Over all, 4S3 shows less tolerance to metals in compare to other two strains (S4 5 and 4S1).

6.11. DISCUSSION

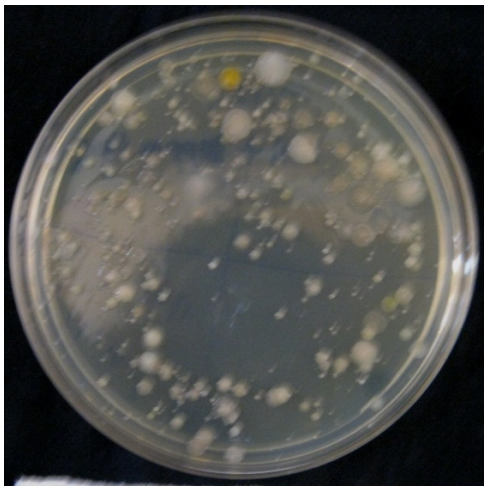
In the present study Nutrient Agar (NA) and Luria Bertani (LB) broth is used for detection of mercury resistant bacteria (MRB). The values of mercury resistant bacteria in the samples ranged from 42% to 84% (Table 1). Mercury resistant bacteria in the present study were isolated by spreading technique on NA plates containing Hg (II). The use of direct selection with high levels of $HgCl_2$ with out prior induction may have inhibited the growth of some mercury resistant bacteria (Osborn et al., 1993).

A total of 20 mercury resistant bacteria from 4 different sites of laggon inlet were isolated on NA medium amended with 10ppm mercury. These 20 strains were then tested for mininmal inhibitory concentration (MIC). 3 of these 20 isolates showed highest MIC and were subjected to 16S rRNA sequencing. These bacteria were capable of growth in medium amended with various heavy metals like $ZnSO_4$, $FeSO_4$, $CdCl_2$, $CuSO_4$. To be noted is that one of the bacteria identified from sediment is *Enterobactericeae* (*Enterobacter* Sp.), so mercury resistance may be a characteristic of the family (Osborn et al., 1993). The results achieved in the study of isolation of mercury resistant bacteria isolated from sediment samples of Rourkela Steel Plant (RSP) were compared with the reported results from other contaminated sediment sites like sediments of Amba estuary. The results reported showed that the values of mercury resistant bacteria in the sediment samples ranged from 3 to 43% while the results achieved in the sediment samples of Rourkela Steel Plant (RSP) showed the percentage mercury resistant bacteria range from 42 to 83.93%. According to these results it is found that the occurrence of mercury resistant bacteria in RSP sediment is higher than that of Ambla estuary. It is found that RSP is polluted and the bacterial strains are more resistant as they undergo selection pressures in the presence of toxic pollutants and develop resistance (Hideomi et al., 1977). Such organisms become important in controlling the basic biological process in contaminated habitats.

In 1998, the concentration of mercury was found to be 0.0017mg/l. The following results show that the concentration of mercury in the sediment is high. As mercury like other trace metals, discharged into water through industrial wastes. It is generally present in association with particulates or has strong affinity for solid phase in the receiving water making sediments its main repository in aquatic environment. Clay minerals, organic and iron and manganese oxides in sediment also influence the concentration of trace metals including mercury in sediments. As RSP is the major source of industrial effluent so it can be easily predicted that the sediments are highly polluted with different metals.

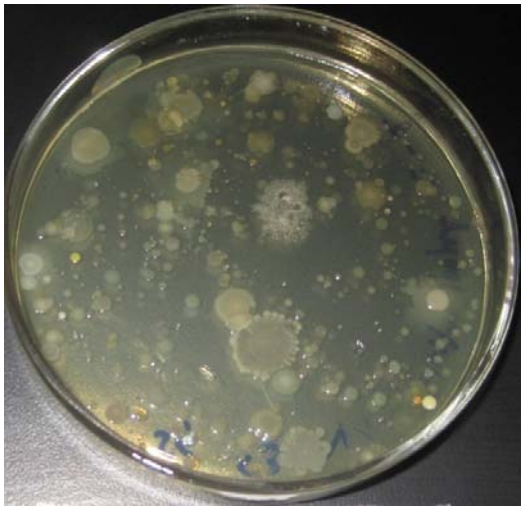
Plate I. Isolated bacterial strains from S1, S2, S3, S4 sites

S1, NA (10^{-2})



S1
NA + 5ppm $HgCl_2$
(10^{-2})

S1
NA + 10ppm $HgCl_2$
(10^{-2})

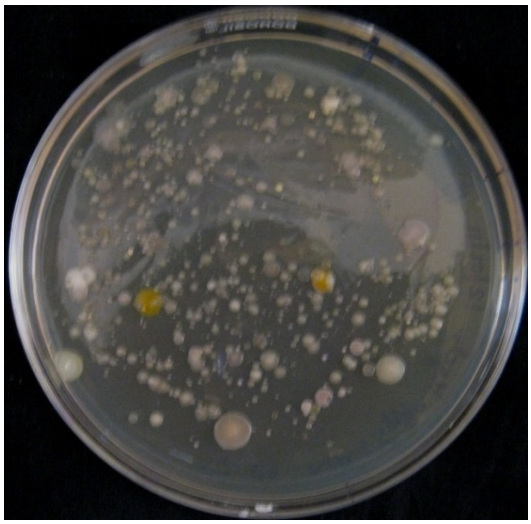


S2, NA (10^{-2})

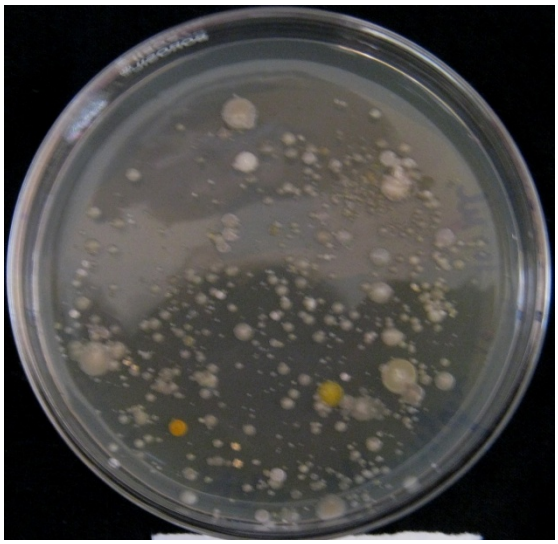
S2
NA + 5ppm $HgCl_2$
(10^{-2})

S2
NA + 10ppm $HgCl_2$
(10^{-2})

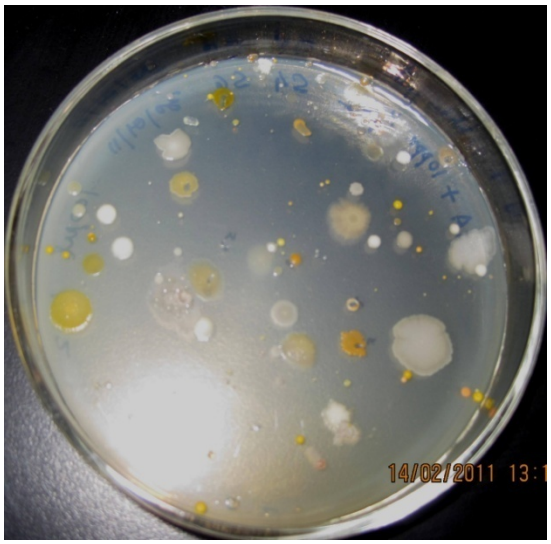
S3, NA (10^{-2})



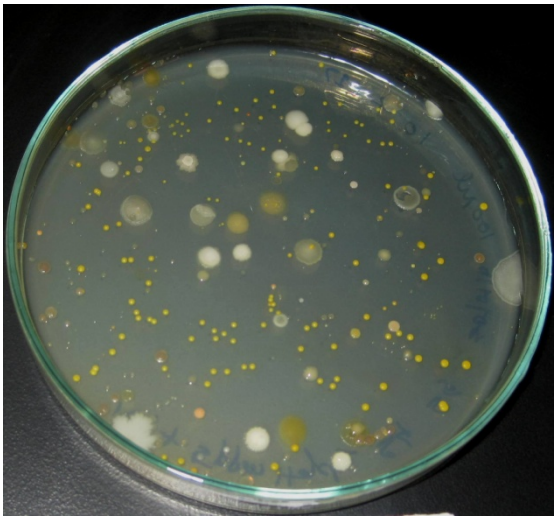
S3
NA + 5ppm $HgCl_2$
(10^{-2})



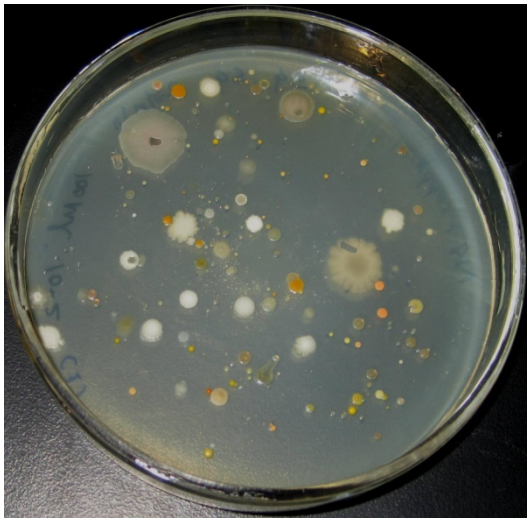
S3
NA + 10ppm $HgCl_2$
(10^{-2})



S4, NA (10^{-2})



S4
NA + 5ppm $HgCl_2$
(10^{-2})



S4
NA + 10ppm $HgCl_2$
(10^{-2})

Plate II. Pure culture of isolated bacterial strains

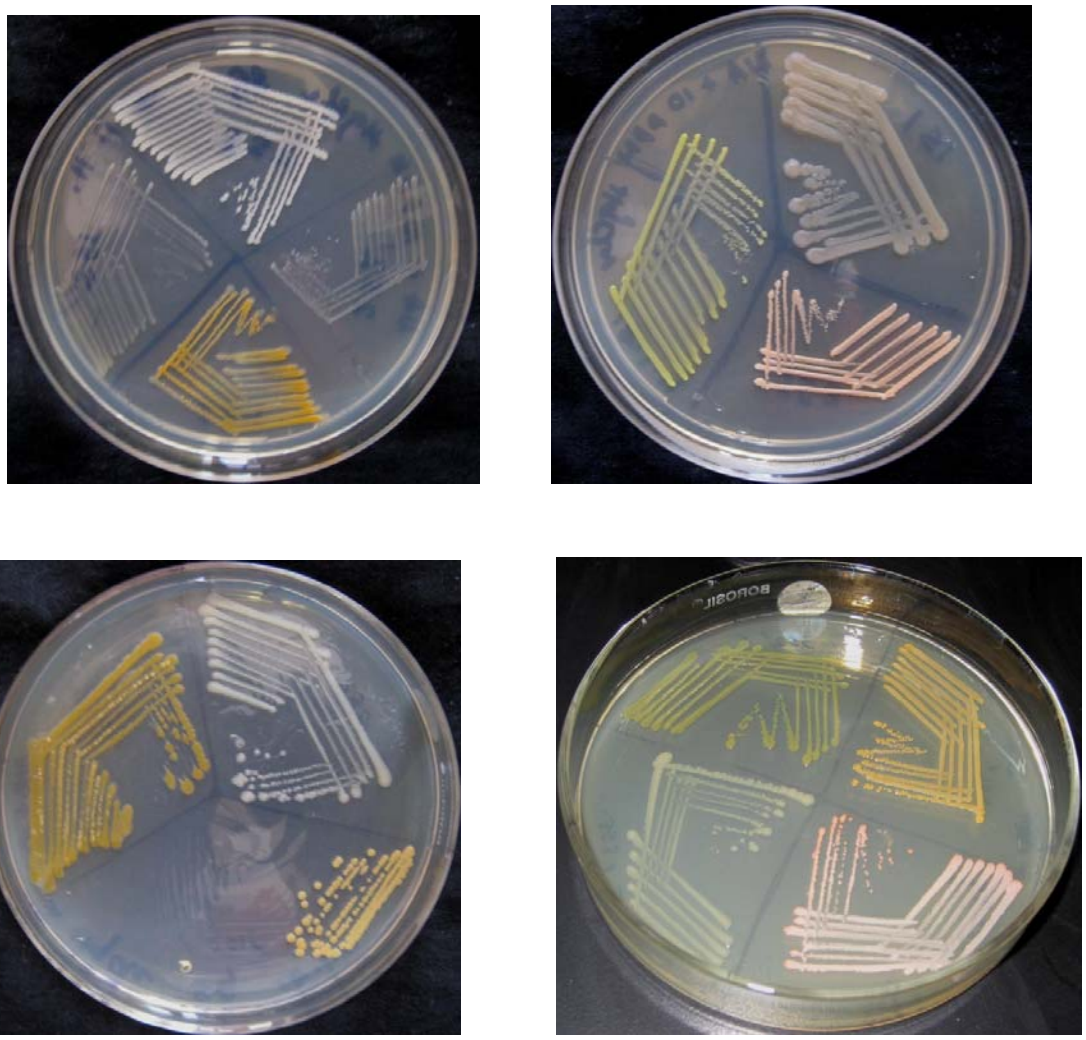


Plate III. Growth on MacConkey's Media

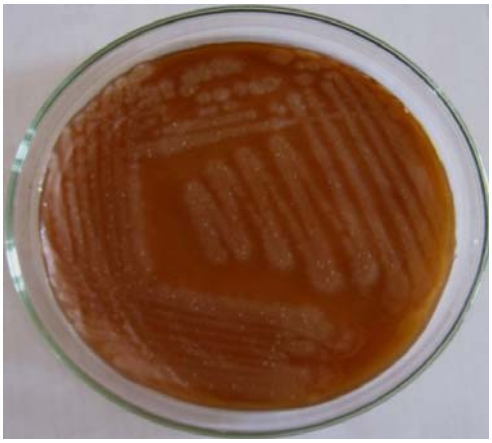
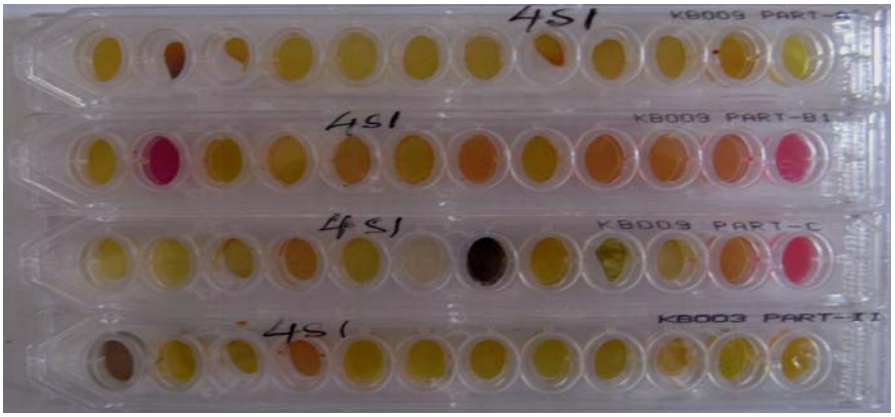
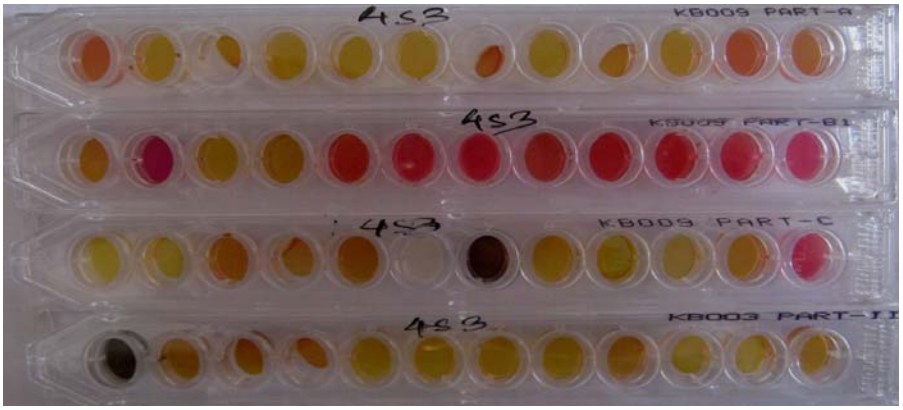


Plate IV. Himedia Rapid Biochemical Identification kit, *Enterobacteriaceae* Identification Kit [KB003 Hi25®]
(A)= Sample 4S1; (B) = Sample 4S3; (C) = Sample S4 5



(A)



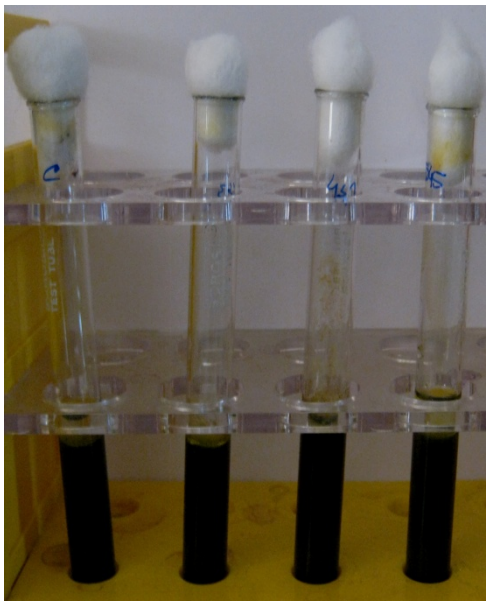
(B)

(C)

Plate V. Biochemical tests

(D) Motility Test:

(E) O/F Basal Media Test:



(F) TSI Test :

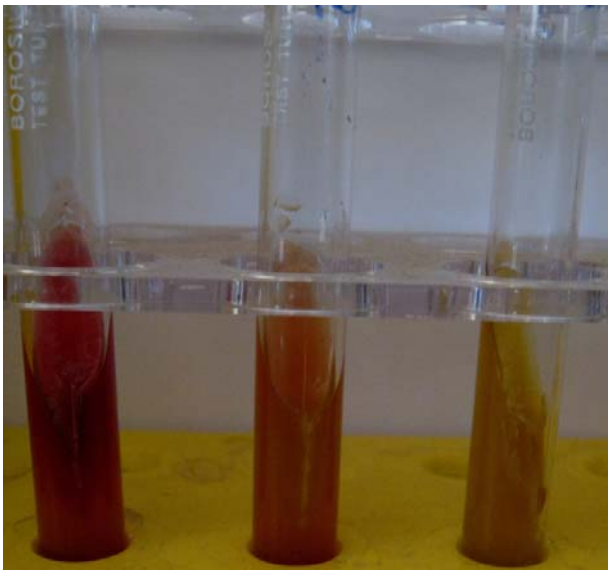
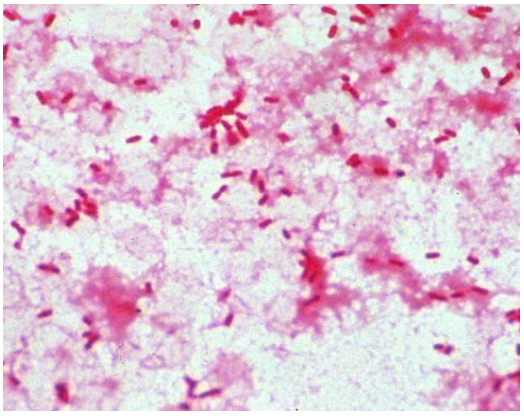
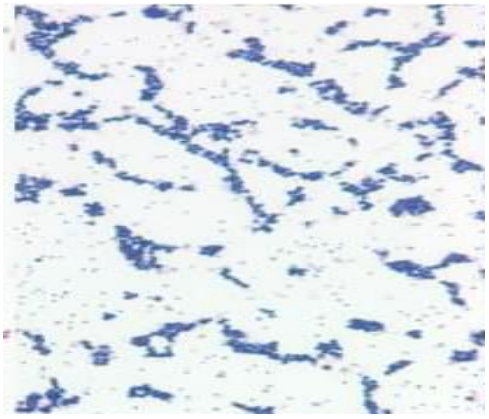


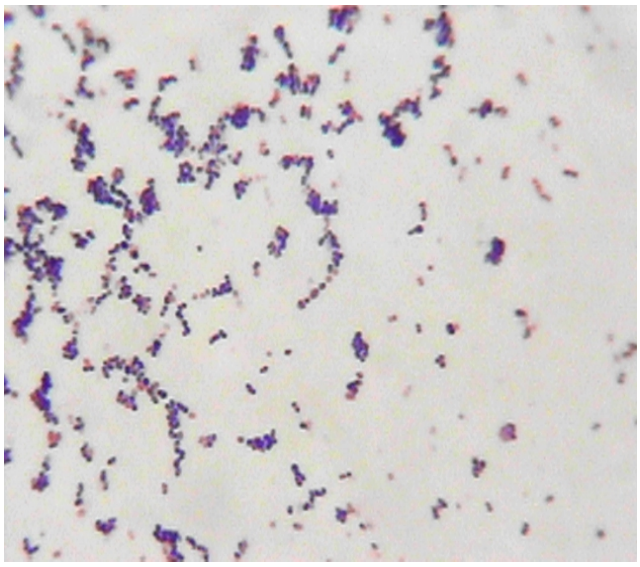
Plate VIII. Gram Staining Results



4S3 : *Pragia fontium*



S4 5 : *Streptococcus canis*



4S1: *Enterococcus saccharolyticus*

7. CONCLUSION:

Reason, Observation and experience: The holy trinity of Science

----------Robert G. Ingersoll

Twenty strains were isolated from four different sites of inlet lagoon of Rourkela Steel Plant, Orissa, of which three strains viz. Streptococcus canis, Pragia fontium and Enterococcus saccharolyticus showed highest minimum inhibtory concentration (MHB). These three strains showed a wide range of resistance to different metals. So these isolates are of interest for molecular characterization of mechanisms for resistance to multiple metals and hold promise for bioremediation of toxic heavy metals, including in environments that are contaminated by several metals. As bacterial mobile genetic elements such as plamids or transposons, can carry multiple genes encoding metal and antibiotic resistance. Mercury resistant bacteria (MRB) isolated from contaminated environments have high potential to remove mercury from factory effluents. So, it is suggested that mercury elimination ability of these bacteria should be evaluated. Moreover these isolates can be genetically engineered to reach better results in removal of mercury.However, before exploiting the strain as an efficient biotechnological tool for mercury detoxification further investigation needs to be carried out in laboratory scale and in-situ metal reduction potential of the genus has to be assessed.

The following conclusions can be withdrawn from the present investigation :

a) Highest percentage of mercury i.e. 83% occurs in site 4 of inlet lagoon from Rourkela Steel Plant (RSP), Orissa.
b) Twenty mercury resistant bacteria were isolated from four different sites of inlet lagoon from Rourkela Steel Plant (RSP), Orissa.
c) Out of twenty strains, three strains viz. *Streptococcus canis, Pragia fontium* and *Enterococcus saccharolyticus* showed highest MIC.

d) Besides mercury, strain S4 5, 4S3 and 4S1 cantolerate to other heavy metals like Zn, Mg,Fe,Cd,Cu.
e) They can grow at different pH concentrations, salinity and temperatures.

7.1. Future Prospects

1. Further investigation on mercury-resistance bacteria may lead to new and better understanding of the existing concept. For instance, presence of non-mer mediated mercury volatilization in the marine bacteria might prove pivotal in acquiring more information on mercury-resistance.

2. Further studies on the role of Na^+ in transport of Hg across the cell membrane and role of Cl^- in determining the bioavailability are quite important in this regard.

3. The resistance of these marine mercury resistant bacteria (MRB) to several heavy metals enthuses to affirmatively recommend their potential to be exploited in bioremediation of mixed wastes. Further studies including on-site experiments will be useful in developing practical means for environmental cleanup.

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APPENDIX-I:

A. MEDIA

The media used and their compositions are given below:

TABLE 1: DETAILS OF MEDIA USED AND THEIR COMPOSITION

1. **NUTRIENT AGAR MEDIA (NA) :**

COMPONENTS	QUANTITY
Beef extract	0.3%
Peptone	0.5%
NaCl	0.5%
Agar agar	1.5%
pH adjusted to 7.2 (at 25°C)	

2. **TSI AGAR :**

COMPONENTS	QUANTITY
Peptone	1%
Tryptone	1%
Yeast extract	0.3%
Beef extract	0.3%
Lactose	1%
Sucrose	1%
Dextrose	0.1%
$FeSO_4$	0.02%
NaCl	0.5%

$NaHSO_3$	0.03%
Phenol red	0.0024%
pH	7.4 ± 0.2
Agar	1.2%

3. OF BASAL MEDIA :

COMPONENTS	**QUANTITY**
Casein enzymic hydrolysate	2.00
NaCl	5.00
Dipotassium phosphate	0.30
Bromothymol blue	0.08
Agar	2.00
pH (at 25°C)	6.8 ± 0.2

4. MANNITAL MOTILITY MEDIUM

5. LURIA BERTANI MEDIA :

COMPONENTS	**QUANTITY**
Tryptone	2.00
NaCl	1.00
Yeast Extract	0.5%
pH (at 25°C)	7.0

6. MacConkey Agar :

COMPONENTS	**QUANTITY**
Peptone	20.0
Trehalose	10.0
Bile Salts (Difco)	1.5
NaCl	5.0
Neutral Red	0.05
Crystal Violet	0.001

Agar	15.0
Distilled water	1.0 (L)
pH (at 25°C)	6.8 ± 0.2

B. STAINS

The bacterial isolates were stained by using Grams staining method:

TABLE 2: COMPOSITION OF GRAM'S STAIN

INGREDIANTS	**USES**
Crystal violet	Primary Staining Agent.
Safranin	Secondary Staining Agent.
Lugol's Iodine	Mordant.
Acetone	Decolourising Agent

APPENDIX-II:

a) $HgCl_2$ SOLUTION:

$HgCl_2$	15 gm
Conc. HCl	2.5 gm

b) KOVAC'S REAGENT